

## **EXHIBIT A**

## Analysis of Aldehyde Dehydrogenase 2 Gene Polymorphism and Ethanol Patch Test as a Screening Method for Alcohol Sensitivity

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TSUTAYA, S., SHOJI, M., SAITO, Y., KITAYA, H., NAKATA, S., TAKAMATSU, H. and YASUJIMA, M. *Analysis of Aldehyde Dehydrogenase 2 Gene Polymorphism and Ethanol Patch Test as a Screening Method for Alcohol Sensitivity.* Tohoku J. Exp. Med., 1999, 187(4), 305-310 — To assess clinical availability of the aldehyde dehydrogenase (ALDH) 2 gene polymorphism to detect alcohol sensitivity among a Japanese population, we determined the ALDH 2 genotypes and also compared to an ethanol patch test in 119 young Japanese. Their alcohol sensitivity was evaluated by a questionnaire on the frequency of alcohol-associated symptoms when they drink. Genomic DNA was extracted from blood samples and amplified by polymerase chain reaction (PCR). PCR primers were flanking the polymorphic region in exon 12 of the ALDH 2 gene. The distribution of the typical homozygote, the heterozygote and the atypical homozygote was 63.9, 31.9 and 4.2%, respectively. Gene frequencies of the typical and atypical alleles calculated from the genotype frequencies were 0.80 and 0.20. The atypical genotypic homozygotes were positively associated with facial flushing symptom, but not with positive response for ethanol patch test. These results indicate that ALDH 2 genotypes determination is essential to detect alcohol sensitivity whereas the ethanol patch test has some limitations. ——— aldehyde dehydrogenase 2 gene; genetics; ethanol patch test; alcohol sensitivity © 1999 Tohoku University Medical Press

Several enzymes including alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are related to the alcohol metabolism. ALDH 2 is a major contributor to alcohol sensitivity and drinking behavior in Orientals (Goedde et al. 1979; Harada et al. 1981). About half of Orientals including Japanese lack mitochondrial ALDH 2 activity, assayed in hair root or liver

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specimens, responsible for the oxidation of acetaldehyde. The persons with deficient ALDH 2 activity show rapid and intense flushing of the face with symptoms of mild to moderate intoxication after drinking alcohol in the amount that has no effect on Caucasoids (Goedde et al. 1983). The facial flushing and other unpleasant symptoms may be due to the absence of ALDH 2 activity and consequent accumulation of acetaldehyde in the body (Goedde et al. 1979; Mizoi et al. 1979). Such alcohol sensitivity might discourage individuals from drinking, thus decreasing the risk of alcohol-related problems including alcoholic liver diseases and acute intoxication of alcohol. Although it would be important to determine the genotypes of enzymes involved in alcohol metabolism to evaluate the genetic backgrounds of alcohol sensitivity, few attempts on the ALDH genotypes and their relevance to alcohol sensitivity have been made in Japanese populations (Shibuya and Yoshida 1988; Takeshita et al. 1994). Ethanol patch test has been employed as a large-scale screening test for alcohol sensitivity because of the simplicity of its methodology, and high sensitivity and specificity to detect it (Higuchi et al. 1987). However, it has been postulated that in the ethanol patch test the proportion of both false positive and negative ranged from 4 to 5% (Higuchi et al. 1987), and some limitations have been revealed for identifying alcohol sensitivity.

Therefore, in the present study, we determined the ALDH 2 genotype among a young Japanese population, and also reevaluated clinical significance of ethanol patch test for identifying ALDH 2 phenotype.

#### MATERIALS AND METHODS

One hundred and nineteen healthy students (male 10; female 109) of Japanese origin aged 18–22 ( $19.9 \pm 1.1$ ) years were selected for the study at School of Allied Medical Sciences, Hirosaki University. Subjects enrollment depended on the normality of clinical characteristics and routine laboratory tests which were performed at our division of laboratory medicine.

##### *Characterization of phenotypes*

To assess alcohol sensitivity, all subjects completed a questionnaire on the frequencies of alcohol-associated symptoms including facial flushing when they drink, according to the previous study (Yamada 1988).

##### *Ethanol patch test*

Ethanol patch test was performed following the method reported previously by Higuchi et al. (1987). Briefly, a patch plaster of two lint pads 15 mm in diameter and 1 mm in thickness, fixed on an adhesive tape was employed in the present study. Just before application of the plaster, 100  $\mu$ l of 70% ethanol was put on one of the lint pads and the same volume of distilled water was put on the other as a control. The patches were attached to the inner surface of the upper

arm for a 7-minutes period and then removed. A patch area which showed erythema 10-15 minutes after removal was judged to be positive.

#### *Determination of ALDH 2 genotypes*

Blood samples were taken for DNA studies with informed consent. Genomic DNA was extracted from whole blood using a DNA extraction kit, SMI TESTR (Sumitomo Bio-Medical, Kashima). The genotype of the ALDH 2 was determined by polymerase chain reaction (PCR) using allele specific primers (Ishibashi et al. 1994). PCR products were separated electrophoretically on 3% agarose gels and DNA was visualized by ethidium bromide staining. PCR products were detectable at the position of 135 bp. ALDH 2 gene polymorphism was classified into three genotypes; the typical homozygous genotype detected only one PCR product by typical genotype detection primers set, the atypical homozygous genotype detected only one PCR product by atypical genotype detection primers set, and the heterozygous genotype detected the PCR band by both primers sets. Data are given as mean and standard deviation (s.d.). Statistical analysis was performed by the  $\chi^2$  test.  $p < 0.05$  was considered statistically significant.

#### RESULTS

The frequencies of the three ALDH 2 genotypes in the subjects are shown in Table 1. The distribution of the typical homozygote, the heterozygote and the atypical homozygote was 63.9%, 31.9% and 4.2%, respectively. Gene frequencies of the typical and atypical alleles calculated from the genotype frequencies were 0.80 and 0.20. Facial flushing was more frequent than other alcohol-associated symptoms, including headache, sleepiness, palpitation, chill, itching, dizziness, muscle weakness, and so on. The frequencies of facial flushing in the typical homozygote and the heterozygote was 36.8% and that in the variant homozygote was 100%, respectively (Table 2). The atypical genotypic homozygotes were positively associated with facial flushing symptom ( $p < 0.01$ ). The

TABLE 1. *ALDH 2 genotype distribution and allele frequency among young Japanese*

|           | Number | (%)  |
|-----------|--------|------|
| Genotypes |        |      |
| NN        | 76     | 63.9 |
| ND        | 38     | 31.9 |
| DD        | 5      | 4.2  |
| Alleles   |        |      |
| N         | 190    | 79.8 |
| D         | 48     | 20.2 |

N, normal allele; D, variant allele.

TABLE 2. *Genotype variants of ALDH 2 in subjects positive or negative for flushing symptom*

|                               | Negative<br>( <i>n</i> = 72) | Positive<br>( <i>n</i> = 47) |
|-------------------------------|------------------------------|------------------------------|
| Genotypes                     |                              |                              |
| NN + ND ( <i>n</i> = 114) (%) | 72 (63.2)                    | 42 (36.8)                    |
| DD ( <i>n</i> = 5) (%)        | 0 (0.0)                      | 5 (100.0)                    |
| Significance                  | <i>p</i> = 0.0047            |                              |

N, normal allele; D, variant allele.

TABLE 3. *Genotype variants of ALDH 2 in negative and positive subjects for alcohol patch test subjects*

|                               | Negative<br>( <i>n</i> = 75) | Positive<br>( <i>n</i> = 44) |
|-------------------------------|------------------------------|------------------------------|
| Genotypes                     |                              |                              |
| NN + ND ( <i>n</i> = 114) (%) | 73 (64.0)                    | 41 (36.0)                    |
| DD ( <i>n</i> = 5) (%)        | 2 (40.0)                     | 3 (60.0)                     |
| Significance                  | <i>p</i> = 0.2759            |                              |

N, normal allele; D, variant allele.

frequency of responsiveness in the ethanol patch test was compared among the ALDH 2 genotypes (Table 3). However, the variant genotypic homozygotes were not positively associated with ethanol patch test.

### DISCUSSION

In the young population of a northern area in Japan, we found ALDH 2 gene frequencies of 0.80 and 0.20 for the typical and atypical alleles respectively, with genotype frequencies of 64%, 32% and 4% for the typical homozygote, the heterozygote and the atypical homozygote. Additionally, in the present study, the apparent differences in the frequency of alcohol-associated symptoms including facial flushing between the atypical homozygote and the other two genotypes were observed, whereas the ethanol patch test does not completely differentiate the atypical homozygote and others.

The frequency of the atypical ALDH 2 allele in a defined Japanese population was in agreement with previous reports (Harada 1990; Goedde et al. 1992; Takeshita et al. 1994) and slightly lower than that reported by Shibuya and Yoshida (1988). There are many reports demonstrating phenotypic differences between those with normal and deficient ALDH 2 activities (Harada et al. 1981; Mizoi et al. 1983; Inoue et al. 1984; Yamada et al. 1988; Muramatsu et al. 1989; Takeshita et al. 1994). Our present results which showing apparent differences in the frequency of the facial flushing between the atypical homozygote and the two other genotypes, reconfirm the previous findings (Takeshita et al. 1994) that the

atypical homozygote is more sensitive to alcohol than the heterozygote. The availability of the method employed for analyzing ALDH 2 genotypes in the present study includes that the atypical homozygotes never evade the acute alcohol-flushing when they drink. Because of limited numbers examined, further studies will be required to confirm the present findings.

The most significant finding in the present study is that the ethanol patch test fails to detect 2 of the 5 atypical homozygotes, whereas it has been regarded as a simple and useful screening test for detection of deficient ALDH 2 activity. Possible explanations for the failure to detect the atypical homozygotes might be due to the difference in skin temperature, the skin color, the absorption of ethanol of individual skins, or the activity of ADH, another contributor of alcohol metabolism (Crabb 1990), however its exact mechanism remains unclear. These results cast the doubt on clinical availability of the ethanol patch test, although further evaluation might be required on the methodology and procedure employed for the ethanol patch test in the present study.

In conclusion, the present study suggests that ALDH 2 genotypes determination is essential to clarify the alcohol sensitivity.

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